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14. ABSTRACT

Loss of estrogen receptor (ER) function has been associated with hyperactive ERK1/2, which culminates in aggressive, radiation resistant cancers. The ERK1/2 pathway has also been linked to DNA damage and repair, with multiple proteins involved in DNA repair being transcriptionally regulated through ERK1/2-dependent signaling. An increased DNA repair capacity in ER-a negative breast tumors has been implicated as a mechanism of radioresistance. We postulate that the mechanism of development of radiation resistance in the ER-a negative breast cancer cells involves a dynamic interplay between the ERK1/2 pathway and DNA repair proteins. We compared ER-a positive and negative cells for expression levels of ERK1/2 and DNA repair proteins involved in the repair of radiation-induced double strand breaks. Preliminary data obtained from clonogenic cell survival assays showed that ER-a positive cells were more radiosensitive compared with the ER-a negative cells. These cell lines are also being compared for the expression of ERK1/2 and its downstream proteins and proteins involved in DNA repair by Western blot analysis. We are also evaluating the ability of inhibitors of the ERK1/2 pathway to restore radiosensitivity to the ER-a negative cell lines. The effect of these inhibitors on expression of DNA repair proteins and their ability to restore ER-a expression will also be tested. The outcome of these studies will have a potential impact in the clinic and benefit breast cancer patients

15. SUBJECT TERMS
None provided.

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Introduction

Breast cancer is the most commonly occurring cancer among women (22% of all cancers in 2000) and is second only to lung cancer as a cause of cancer deaths in women (15% of cancer deaths) (1, 2). The estimated annual incidence of breast cancer worldwide is about one million cases with ~200,000 cases in United States (27% of all cancers in women) and ~320,000 cases in Europe (31% of all cancers in women) (3, 4). Over the last two decades, the annual incidence rate in US has been increasing steadily (5). Women with an early diagnosis and favorable risk factors are cured by primary surgical and radiotherapy treatment while those with more advanced or aggressive tumors experience recurrence and later death (5). Risk factors for recurrence are generally related directly or indirectly to the rate of cell proliferation and the percentage of cells undergoing apoptosis. The factors controlling these two interrelated processes are complex and not fully understood.

Radiotherapy of patients with breast cancer remains an important cancer treatment modality and plays an essential role in local and regional control of the disease (6). It has been estimated that more than 50% of all cancer patients receive radiation as part of their overall management. Randomized trials have demonstrated the efficacy of radiation therapy in the treatment of breast cancer. Even though many of these patients benefit from their treatment, between 30-50% of patients with localized disease initially fail at their primary tumor sites following therapy. A variety of strategies have been and are continuing to be actively explored to improve local control. Tumors locally fail after radiation therapy due to biological factors associated with the particular tumor. Advances in our knowledge of the molecular pathways that govern some of these factors has generated many new ideas that can be explored for improving the efficacy of radiation therapy but there are still aspects of tumor sensitivity to radiation that are poorly understood (7-9).

Since radiation therapy plays a critical role in the management of a majority of breast cancer patients, identification of factors that help predict which patients are at risk for relapse within the irradiated field remains an active area of investigation. A substantial amount of research has been devoted to identifying predictive markers for radiation resistance. Loss of estrogen receptor (ER) function has been associated with constitutive and hyperactive MAPK (particularly ERK1/2), which culminates in aggressive, metastatic, radiation-resistant cancers. Activation of the ERK1/2 cascade modulates the phosphorylation and activity of several nuclear transcription factors that in turn regulate a series of genes involved in promoting cellular survival and resistance to chemotherapy and ionizing radiation. The ERK1/2 pathway has also been linked to DNA damage and DNA repair, with multiple proteins involved in DNA repair being transcriptionally regulated through ERK1/2-dependent signaling (10-21). An important hallmark that dictates the radioresistant phenotype of tumor cells and is probably the most critical factor in the radiation responsiveness of a tumor is the ability of a cancer cell to repair and recover from radiation-induced DNA double-strand breaks (DSBs). An increased DNA repair capacity in ER- α negative breast tumors has also been implicated as a mechanism of radioresistance. We postulate that the mechanism of development of radiation resistance in the ER- α negative breast cancer cells involves a dynamic interplay between the ERK1/2 pathway and DNA repair proteins.

Body:

Breast cancer is a heterogeneous disease, displaying wide variances in response to various therapeutic approaches and outcome. Generally, hormone receptor negative tumors are high grade, poorly differentiated tumors. In accordance with these observations, decreased survival rates are reported for patients with estrogen- or progesterone-receptor negative tumors compared to those with hormone receptor positive breast cancer (22, 23).

The epidermal growth factor receptor (EGFR)/Her-2/neu/Ras/MEK/mitogen activated protein kinase (MAPK) and the c-kit-Akt / PI3K (phosphoinositol-3-kinase) pathways are two major signal transduction pathways that lead to activation of intracellular driving mechanisms for proliferation and antiapoptotic features of tumor cells. It has been previously demonstrated that MAPK family members, including ERK, JNK and p38 MAPK play an active role in the proliferation, invasive capacity and generation of metastatic potential for cancer cells, as well as chemoresistance (10-21). Furthermore, the MAPK family has been shown to have a regulatory role in providing the complex balance between cellular growth and death through competing

interactions. Therefore, the exact mechanism by which MAPK is involved in the pathogenesis of breast cancer is not clear and remains to be elucidated further.

Intracellular signaling through the Ras-MAPK pathway has been observed in a wide range of breast tumors and has been linked to non-genomic estrogen- mediated tumor growth and induction of estrogen receptor-negative phenotype, in addition to resistance to hormonal agents, such as tamoxifen (24-33). MAPK overexpression has also been associated with growth factor related and anchorage-independent tumor proliferation by increased heat shock protein expression in triple negative tumors and is in concordance with in vitro data suggesting that active MAPK signaling is correlated with estrogen receptor negativity and induction of receptor negative phenotype (24-33). The role of MAPK has not been extensively evaluated in a prospective trial, and data available is generally limited to analysis of archival material.

We postulate that the mechanism of development of radiation resistance in the ER- α negative breast cancer cells involves a dynamic interplay between the ERK1/2 pathway and DNA repair proteins.

Aim 3: Generation of tissue arrays and immunohistochemical analysis of patient specimens for expression of DNA repair proteins and signaling intermediates in the ERK pathway.

i). We will evaluate the prevalence of the ERK pathway and its downstream targets, as well as DNA repair proteins (BRCA1, BRCA2, DNA-PK, GADD-45 and Topo-II α) in a cohort of clinical breast cancer specimens previously used to investigate for markers of locoregional failure after radiation therapy. An attempt will be made to correlate loss of ER-α with hyperactive ERK1/2 and high levels of DNA repair proteins in clinical samples. The samples will be analyzed by tissue microarray. (Months 24-36).

Key Research Accomplishments

The progress made towards each sub-specific aim is briefly summarized in this section.

In our previous report we had compared the intrinsic radiosensitivity of a panel of human breast cancer cell lines and shown that cell lines expressing estrogen receptor (MCF-7) were more sensitive to increasing doses of radiation when compared with the ER negative cells (MDA-MB-231, MDA-MB-453, MDA-MB-435 and Hs578t). ER- α negative cell lines had higher SF2 values when compared with the ER-α positive MCF-7 cells indicating intrinsic radioresistance of ER-α negative cells. In addition we tested MDA-MB-231 cells that were stably transfected with full length ER-α (clones designated ERα-3 and ERα-6). MB231 cells transfected with vector backbone were used as controls (designated LxSN2 and LxSN23). The estrogen receptor expressing ERα-6 clone was more sensitive to increasing doses of radiation when compared with the vector control cells. The survival enhancement ratio was enhanced when the estrogen receptor gene was put back into the cells. Both the cell lines were also compared for the level of expression of ER- α by western blot analysis. Following these experiments, we compared the basal levels of activated ERK1/2 and levels of DNA repair proteins (NBS1, RAD51, and Topo-IIα) in ER-α negative (MDA-MB-231, MDA-MB-468, MDA-MB-435 and Hs578t) and ER- α positive (MCF-7 and ZR75-1) breast cancer cell lines by Western Blot Analysis. ER- α negative cells had higher levels of phosphorylated ERK and DNA repair proteins such as phospho-NBS1 and RAD51. Levels of Topo-II α were also higher in ER-α negative breast cancer cell lines. However ZR75-1, an ER-α positive cell line, also expressed high levels of Topo-II α.

We examined a panel of human breast cancer cell lines for estrogen receptor- α expression by western blot analysis. The panel included ER- α positive (MCF-7, ZR75-1 and T47D) and ER- α negative (MDA-MB231, MDA-MB468 and MDA-MB-435) cell lines. Since over-expression of EGFR is inversely correlated with ER-α we also looked for EGFR expression in the cell lines mentioned above by Western blot analysis. ER-α negative cell lines had high expression of EGFR compared to the ER-α positive cells.

Since transient/con stitutive expression of MAPK leads to downregulation of ER- α we obtained an MCF-7 breast cancer clone engineered to overexpre ss EGFR and thereby activat ed phospho-MAPK/ERK. In this cell line, designated as MCE-5, we compared the levels of pERK and ER- α in MCE-5 and MDA-MB-231 cells. The MCE-5 cells had a higher constitutive level of pE RK when com pared to MCF-7 cells. Exposure to 5Gy dose of radiation led to an incr ease in ERK leve ls in th e MCF-7 ce lls but not in the MCE-5 or the MD A-MB-231 cells. Imm unohistochemical analysis was also perform ed on the Hs578t, MDA-MB-231 and the MCE-5 cells for activated ERK. MDA-MB-231 and Hs 578t cells showed positive staining for ERK. The MCE-5 cells overexpressing activated ERK however were very strongly positive for ERK by immunohistochemistry.

Since ERK is constitutive ly active in Hs578t and MDA-MB-468 cel ls as detected based on phospho-p44/p42 expression, we have tested the ability of the ERK inhibitor U0126 to radiosensitize ER-α negative cells. U0126 was found to res tore radiation sens itivity to Hs 578t, ER- α negative cells, which ar e known to be extrem ely radioresistant. Sim ilar results were obtained with MDA-MB-468 cells. Additi onally we found that MCF-7 cells which do not constitu tively express ERK are not radiosensitized by U0126 indica ting that the E RK pathway does not mediate the radiation sensitivity of these cells.

In the last report we had prepar ed MDA-MB-231 stable clones in which we used shRNA to knockdown expression of activated ERK1/2. These stab le clones were characterized for dow nregulation of pERK1 or pERK2 by western blot analysis and then tested for their response to radiation. MDA-MB -231 clone with ERK1 knockdown was more sensitive to ra diation when compared to the cont rol transfected cells. The degree of sensitization was less than what we have obtained with U0126 but that could be attributed to the fact th at U0126 downregulates both ERK1 a nd ERK2 whereas in the shRNA clone we are knocking down either ERK1 or ERK2.

However, since the last report we have had to go back and prepare these stable clones again as the insert was lost in the previous cell lines. W e have analyzed these clones for di fferences i n radiation se nsitivity and the data is shown in Figure 1. Knockdown of both ERK1 and ERK2 rad iosensitized MB231 cells to a gre at extent.

We also carried out a cell cycle analys is on these clones and found that knockdown of ERK2 (MAPK1) blocked the cells from entering into G2 phase following 5Gy dose of radiation when compared with sh-control transfected cells.

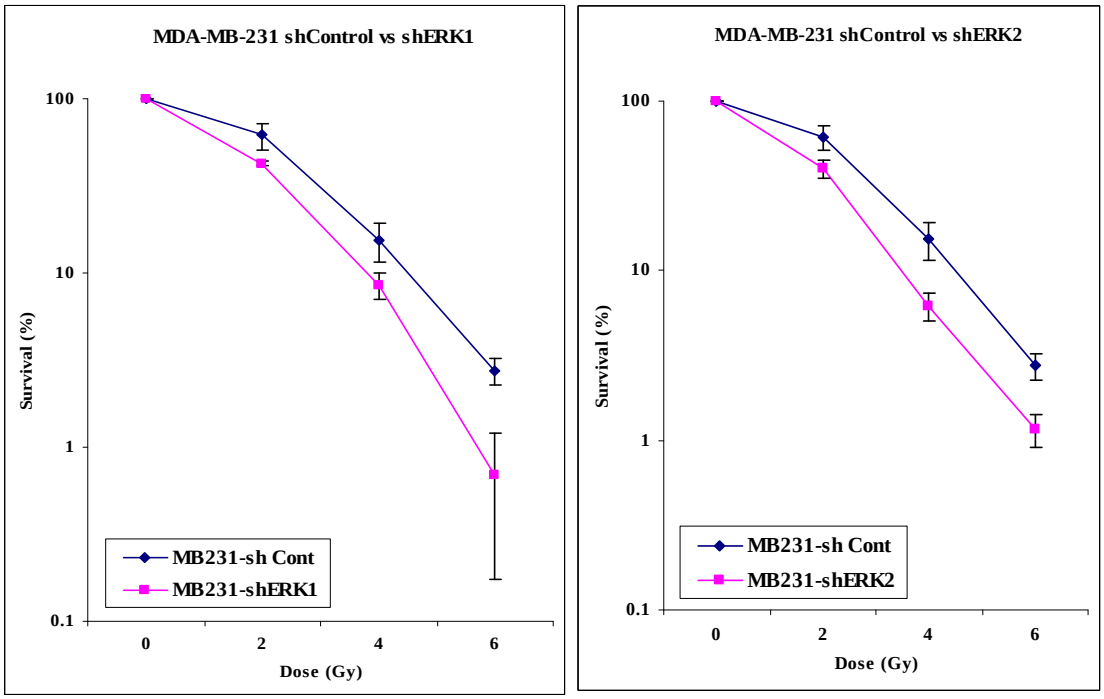


Figure 1: shRNA to ERK1 and ERK2 was used to downregulate ERK in MDA-MB-231 cells and associate loss of ERK1/2 to radiation sensitivity.

However, shERK1 (MAPK3) enhanced the G2 block compared to the controls (Figure 2).

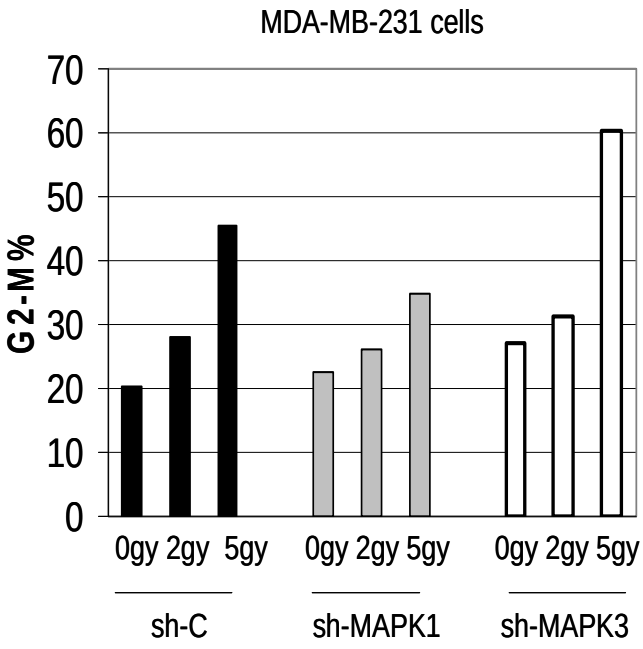
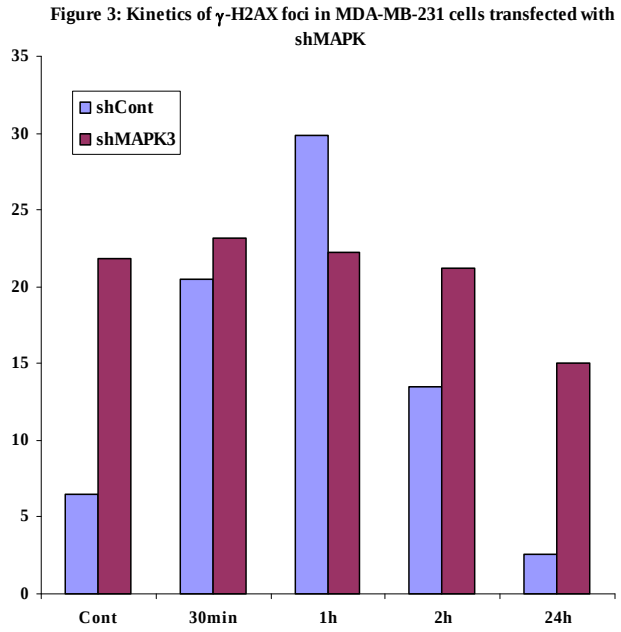


Figure 2: Cell cycle distribution of ERK downregulated cells following exposure to radiation.



We also analyzed these clones for their DNA repair capacity by studying the kine tics a gamma H2AX fo ci formation following exposure to 2Gy dose of radiation. As can be seen from figure 3, the shMAPK3 clones had more number of foci to begin with and the foci were prolonged for a longer period of time when compared with

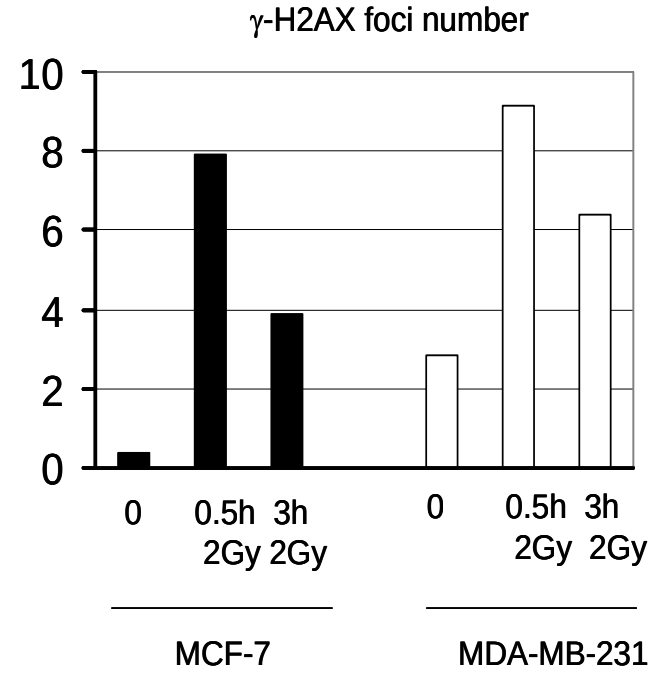


Figure 4: MB231 cells have prolonged expression of g-H2AX foci when compared to the MCF-7 cells

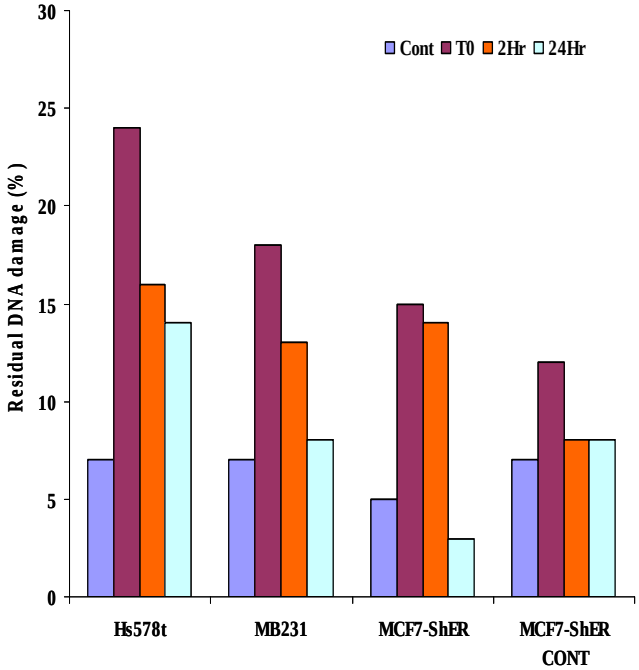


Figure 5: Comparison of γ-H2AX foci in cell lines with and without estrogen receptor expression

the control cells. Similar results were also obtained for the shMAPK1 clones.
We also obtained sim ilar results when we com pared the ER negative MDA-MB- 231 cells with ER-positive MCF-7 cells or MCF-7 in which ER has been downregulated (Figure 4 and 5).

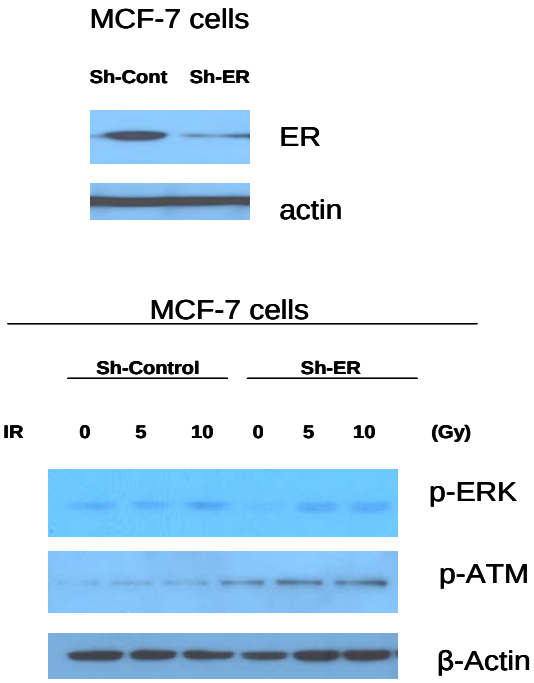


Figure 6: Downregulating ER activates ERK and ATM following exposure to radiation

MCF-7 cells in which ER e xpression was knocked down with shRNA also showed an enhan ced activation of pATM upon radiation ex posure when com pared with the shControl ce lls (Figure 6).

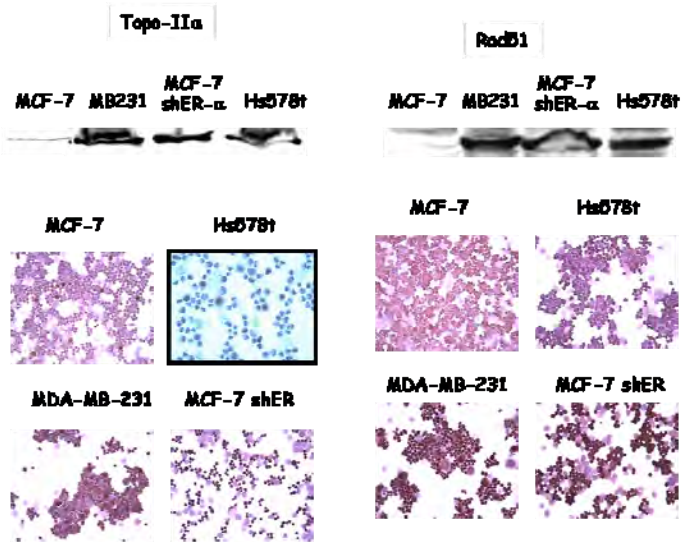


Figure 7: ER-α negative breast cancer cells have high levels of activated ERK and DNA repair proteins compared to ER-α positive cells

As the m ajor part of this aim was d irected towards immunostaining for these DNA re pair proteins we have spent a lot of ti me in standardizing our staining protocol. We standardized the stain ing protocol on 3 different cells lines with varyin g estrogen recepto r status (M DA-MB-231: ER negative; MCF-7 shER: with estrogen receptor knockdown; and MCF-7: estrogen receptor positive). However the staining had to be re-standardized on paraffin embedded cell blocks and on mock tissue arrays. Because of these delays we have requested for an extension of the project for a period of 6 months so that we can com plete the staining on the TMA and analyze the data obtained.

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